Measuring Epstein–Barr virus (EBV) load: the significance and application for each EBV-associated disease

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SUMMARY

Because Epstein–Barr virus (EBV) is ubiquitous and persists latently in lymphocytes, simply detecting EBV is insufficient to diagnose EBV-associated diseases. Therefore, measuring the EBV load is necessary to diagnose EBV-associated diseases and to explore EBV pathogenesis. Due to the diverse biology of EBV, the significance of measuring EBV DNA and the optimal type of specimen differ among EBV-associated diseases. Recent advances in molecular technology have enabled the EBV genome to be quantitated rapidly and accurately. Real-time polymerase chain reaction (PCR) is a rapid and reliable method to quantify DNA and is widely used not only as a diagnostic tool, but also as a management tool for EBV-associated diseases. However, each laboratory currently measures EBV load with its own “homebrew” system, and there is no consensus on sample type, sample preparation protocol, or assay units. The EBV real-time PCR assay system must be standardised for large-scale studies and international comparisons.

INTRODUCTION

Epstein–Barr virus (EBV) belongs to the genus Lymphocryptovirus, subfamily Gammaherpesvirinae, family Herpesviridae [1]. In primary infection, EBV is predominantly asymptomatic but occasionally causes infectious mononucleosis in adolescents or young adults. Rarely, chronic active EBV infection develops in immunocompetent hosts [2,3]. Moreover, several malignancies, including Burkitt’s lymphoma, Hodgkin’s lymphoma, nasal natural killer (NK) cell lymphoma, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorder (PTLD) have been etiologically linked to EBV infection [2,3].

Because EBV is ubiquitous and establishes a lifelong persistent infection after primary infection, simply detecting EBV is insufficient to diagnose EBV-associated diseases. Measuring the EBV load is essential to follow and diagnose EBV-associated diseases and to explore the pathogenesis of EBV infection. Over the last decade, advances in molecular technology have enabled minimal amounts of DNA to be quantified rapidly and accurately, and such techniques have been used to diagnose viral infection. For EBV infections, a variety of methods, techniques, and protocols have been used to measure EBV loads at many institutions.

However, EBV detection techniques and viral load estimation values have not been standardised and results vary between different laboratories [4]. Furthermore, there is no consensus regarding
what type of samples should be tested: peripheral blood mononuclear cells (PBMCs), whole blood, plasma, or serum. In this review, we summarise the principles of measuring EBV load based on the biology of EBV infection and propose protocols for managing EBV-associated diseases.

**BIOLOGY OF EBV INFECTION**

Similar to other gammaherpesviruses, EBV establishes a life-long infection in B cells. Figure 1 shows a schematic representation of both primary and persistent EBV infections. In primary infection, cell-free EBV in the saliva infects naïve B cells in the oropharynx [5]. B cells are infected after the viral envelope glycoprotein, gp350/220, attaches to the cell surface protein CD21, the primary EBV receptor [6]. EBV initiates a latent growth-transforming infection, causing naïve B cells to transform into proliferating blasts. In immunocompetent hosts, both EBV-specific cytotoxic T lymphocytes (CTL) and NK cells control the outgrowth of EBV-transformed cells during primary infection [7]. Primary EBV infection is usually asymptomatic, but occasionally progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [7]. EBV then establishes a latent infection in memory B cells (Figure 1), which are non-permissive for viral replication [8,9]. After convalescence, EBV persists latently in these memory B cells in an episomal form. These virus-infected cells persist at a low level, approximately 1 in 10,000 to 100,000 memory B cells [5]. Occasionally, infected memory B cells differentiate into plasma cells that undergo lytic infection and produce virus (Figure 1). Newly infected naïve B cells have phenotypes of transformed cells, but are controlled by CTL unless immunity is suppressed. In immunocompromised hosts, transformed cells become proliferating blasts that can result in symptomatic disease, such as PTLD.

The epithelial cells of Waldeyer’s ring are also infected by EBV and shed virus during primary infection [10,11]. EBV replicates in a permissive cell type in the oropharynx, probably specialised epithelial cells, that either binds virus directly or acquires virus by transfer from the surface of adjacent B cells (Figure 1) [12]. EBV infects epithelial cells through a CD21-independent mechanism, and the viral glycoprotein gH mediates EBV attachment to CD21-negative epithelial cells [13].

Figure 1. Schematic representation of Epstein–Barr virus (EBV) infection. In primary infection, EBV in the saliva directly infects naïve B cells in the oropharynx. EBV-infected B cells transform and proliferate as activated blasts but are finally controlled by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells. After convalescence, EBV persists as a latent infection with episomal DNA in memory B cells. Occasionally, memory B cells differentiate into plasma cells that undergo lytic infection and produce virus. Newly infected naïve B cells become transformed, but are controlled by CTL unless cellular immunity is suppressed.

Accumulating evidence suggests that EBV also infects T and NK cells during primary infection. Although T and NK cells do not typically express CD21, it is expressed by thymic T cells [14]. In the tonsils of acute infectious mononucleosis patients, EBV-positive T and NK cells are seen, although they are rare [15,16]. In addition, T and NK cells are detected in the peripheral blood during acute infectious mononucleosis [17]. Recently, Isobe et al. reported the in vitro infection of human NK cells by EBV [18]. Moreover, NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV to bind to NK cells [19].

**CLASSIFICATION OF EBV-ASSOCIATED DISEASES BY EBV LATENT GENES**

In healthy individuals, EBV is latently maintained in memory B cells, which express only the transcripts for EBV-encoded small RNAs (EBERs) [20,21]; this state is termed latency 0 (Table 1) [1,22]. In EBV-associated diseases, viral gene expression is classified into one of the three other latency patterns [1,2]. In latency type I, which is found in Burkitt’s lymphoma [23], EBV nuclear antigen (EBNA)-1 and BamHI A rightward fragments (BARTs) are expressed in addition to EBERs (Table 1). In latency type II, characteristics of Hodgkin’s lymphoma [24] and nasopharyngeal carcinoma [25], EBNA-1, latent membrane protein (LMP)-1, LMP-2, BARTs, and EBERs are expressed (Table 1). In latency type III, associated with lymphoproliferative disorders [26], all latency genes, including EBNA-2 and EBNA-3s, are expressed (Table 1). As EBNA-3s are dominant CD8+ CTL targets [7], cells in latency type III are usually eliminated by CTL. Thus, latency type III is only maintained in immunosuppressed states, such as in post-transplant or AIDS patients. On the other hand, in latency types I and II, only a restricted number of less-antigenic EBV latent genes are expressed, allowing EBV-infected cells to evade CTL [7].

Although EBV latency patterns can be classified grossly into these four types, this classification is not very strict, and heterogeneous patterns are reported in EBV-associated diseases [27,28]. Patterns of viral gene expression can differ between different cell subsets in the same individual or even tissue. Moreover, both latent and lytic infections are observed within the same patient or tissue. For example, in infectious mononucleosis, EBV-transformed B cells undergo latency type III, while plasma cells or epithelial cells are in lytic infection and produce cell-free virus. In nasopharyngeal carcinoma, only a few cells may enter lytic viral replication [29], while the majority of nasopharyngeal carcinoma cells are in the latent II phase [25].

**TECHNICAL ASPECTS IN MEASURING EBV LOAD IN PERIPHERAL BLOOD**

Several methods have been developed to measure EBV load and are summarised in Table 2. When possible, detecting virus-associated antigens using virus-specific antibodies is a direct

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### Table 1. Patterns of Epstein–Barr virus (EBV) infection and EBV-associated diseases

<table>
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<tr>
<th>Pattern</th>
<th>EBV-related genes</th>
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<td>EBNA-1</td>
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<td>Latency I</td>
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<td>Latency III</td>
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EBNA, EBV nuclear antigen; LMP, latent membrane protein; BARTs, BamHI A rightward transcripts; EBERs, EBV-encoded small RNAs; NK, natural killer.

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and easy way to measure viral load in the peripheral blood. However, there are currently no good monoclonal antibodies or suitable antigens for EBV. Only one antigen, EBNA-1, is expressed in all EBV-associated diseases. As the EBNA-1 protein is expressed at low levels in EBV-infected cells, an anti-complement immunofluorescence method is required to enhance the fluorescent signal. However, the sensitivity of this method is too low to be applied routinely in clinical settings (Table 2).

EBER-1, one of the EBERs, is detectable in virtually all EBV-infected cells and is expressed at very high levels, reaching $10^7$ molecules per cell, although no protein is apparently translated. Previous studies have used in situ hybridisation with an EBER-1 probe to detect and count EBV-infected cells [30,31]. This technique is widely used to detect EBV in tissue specimens [32]. Although EBER-1 in situ hybridisation is a specific and direct method to detect EBV-infected cells, it is only applicable to infected cells and does not detect cell-free virus (Table 2). Furthermore, specialised skills are needed to handle RNA. This technique is not currently used to measure EBV load in the peripheral blood, and in situ hybridisation has been replaced by methods that detect amplified DNA, such as polymerase chain reaction (PCR).

PCR is a sensitive and rapid DNA detection method that has been used to measure EBV loads. Semi-quantitative PCR, by endpoint detection of diluted samples or by quantifying amplified products, was first developed in the mid-1990s [33–37]. However, the linear range of such semi-quantitative PCR is too narrow to measure a variety of samples because the amount of amplified product reaches a plateau after the log phase of the reaction [38]. This inaccuracy limits this method to the detection of only very large differences (Table 2) [39]. To overcome this problem, quantitative-competitive PCR, which uses the presence of co-amplified PCR targets of known concentrations, was developed in the late 1990s [40,41]. The competitor acts as a standard and as a control for differences in amplification efficiency and enables quantitative-competitive PCR to determine EBV loads within two- to four-fold differences [39]. However, quantitative-competitive PCR requires both time and skill to complete as this assay includes gel electrophoresis and Southern blot hybridisation steps (Table 2). Thus, this method has not been widely used.

Real-time PCR is a rapid and reproducible method for quantifying DNA that was first introduced for EBV in 1999 [31,42–44]. Real-time PCR measures the accumulation of PCR products with either a fluorogenic probe or SYBR green I dye, coupled with real-time laser scanning. In the former system, a dual-labelled fluorogenic hybridisation probe (a “TaqMan” probe) is commonly used. One fluorescent dye serves as a reporter and its emission spectrum is quenched by the second fluorescent dye. Nuclease degradation of the hybridisation probe releases the quenching of the

<table>
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<td>Immunofluorescence</td>
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<td>Low sensitivity</td>
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<td>EBER-1 in situ hybridisation</td>
<td>RNA</td>
<td>Moderate</td>
<td>Slow</td>
<td>Difficult</td>
<td>Fair</td>
<td>No good antibodies available</td>
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<td>Requires specialised skills</td>
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<td>Semi-quantitative PCR</td>
<td>DNA</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Poor</td>
<td>Inaccurate quantification</td>
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<tr>
<td>Quantitative-competitive PCR</td>
<td>DNA</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Fair</td>
<td>Requires time and labour</td>
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<tr>
<td>Real-time PCR</td>
<td>DNA</td>
<td>High</td>
<td>Rapid</td>
<td>Easy</td>
<td>Fair</td>
<td>Needs special equipment</td>
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EBER-1, Epstein-Barr virus encoded small RNA 1; PCR, polymerase chain reaction.

Table 2. Representative methods to measure Epstein–Barr viral load

reporter fluorescent emission, resulting in an increase in peak fluorescence [45]. In the latter system, SYBR green I dye is used as a marker for product accumulation. This system is less expensive, but less specific for EBV than a hybridisation probe strategy [46].

Real-time PCR has a large dynamic range for target molecule determination because real-time measurement of the PCR product enables the amplified products to be quantified in the log phase of the reaction [45]. Furthermore, because the reaction is performed and measured in sealed wells, the system does not require the many precautions that are taken with amplified products to avoid contamination. This is a great improvement over conventional PCR assays, which have considerable risks of carry-over contamination. With its speed, accuracy, and ability to handle many samples, the real-time PCR assay has replaced other quantitative PCR methods and is now widely used for measuring EBV load (Table 2). One disadvantage of real-time PCR is the need of specialised and relatively expensive equipment for real-time laser scanning, although the cost is decreasing. Like other EBV methods, there is currently no standardised real-time PCR protocol for measuring EBV load [4]. To date, all real-time PCR assays used to measure EBV load have been “in house” or “homebrew” systems, and primers and probes differ across many laboratories. Most important, the real-time PCR assay requires a standard, usually a plasmid containing the target gene. As these standards are made and serially diluted in individual laboratories, EBV values from each system cannot be compared, even when the same system is used.

APPLICATION OF EBV LOAD MEASUREMENTS FOR EACH EBV-ASSOCIATED DISEASE

The biology of EBV infection is complex and differs across the EBV-associated diseases. For example, in PTLD, blast B cells in latency type III proliferate and migrate into the peripheral blood. Most EBV genomes in the peripheral blood are cell-associated (Figure 2). In contrast, in nasopharyngeal carcinoma, malignant cells proliferate in tissues and rarely migrate into the peripheral blood, and most EBV genomes in peripheral blood are cell-free. Therefore, determining the EBV load depends on whether and how much cell-asso-

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Infectious mononucleosis/EBV-associated haemophagocytic syndrome

In primary EBV infection, such as infectious mononucleosis, both cell-associated and cell-free EBV exist in the peripheral blood (Figure 2) [47]. Some transformed B cells in latent infection leak into the bloodstream. Encapsidated viral genomes (virions), which are produced from plasma cells in lytic infection, flow into the peripheral blood [48]. Fragmented or naked DNA from apoptotic cells may also be detected as cell-free EBV DNA in the peripheral blood. Thus, EBV DNA can be detected in both cell-free blood (serum or plasma) and PBMCs.

In primary EBV infection, EBV DNA is detected in the blood of virtually all patients [35]. After the appearance of EBV-specific immunity, EBV is controlled [49], and the EBV DNA load decreases gradually in PBMCs but disappears rapidly from the plasma [50–52]. Memory B cells, in which EBV is latent in an episomal form, remain in the peripheral blood. As EBV DNA is detected infrequently in the serum or plasma of healthy seropositive carriers [35,53,54], the presence of cell-free EBV DNA signifies a primary infection or reactivation of EBV. These observations indicate that plasma and serum are the most desirable specimens for identifying infectious mononucleosis or primary EBV infection (Table 3).

As infectious mononucleosis is a self-limiting disease that is usually diagnosed by clinical symptoms and serology, measuring EBV DNA is not necessary for the diagnosis. However, EBV load in the serum or plasma correlates with disease severity in infectious mononucleosis [35,52,55]. Primary EBV infection occasionally causes haemophagocytic syndrome. This disease, also called haemophagocytic lymphohistiocytosis, is rare in Western countries, but is common in eastern Asia [1,56]. As EBV-associated haemophagocytic syndrome can be a severe and even life-threatening disease, early diagnosis and intensive therapy are necessary [56]. Extremely high viral loads are seen in both PBMCs and the serum of patients with EBV-associated haemophagocytic syndrome [35,50,55,57]. Monitoring EBV DNA in serum is useful for evaluating the therapeutic response [55,57].

Post-transplant lymphoproliferative disorder

In immunosuppressed transplant patients, EBV-transformed B cells become proliferating blasts and occasionally progress to PTLD. Transformed B cells proliferate vigorously in lymphoid tissues and migrate into the peripheral blood. Most EBV is cell-associated and EBV DNA is detected in high copy numbers in PBMCs (Figure 2).
Cell-free, non-encapsidated EBV DNA is also detected in the blood, indicating fragmented or naked DNA [48]. Riddler et al. were the first to show that molecular testing for EBV in the peripheral blood could be used to non-invasively monitor PTLD [34]. Measuring EBV DNA has since been studied extensively and is now an indispensable tool for controlling PTLD. This method is used not only for diagnosis, but also for disease prediction [33,34,58–60], therapeutic efficacy estimation [61,62], and prevention [63–66].

There has been debate regarding which specimen(s) should be used to identify PTLD. Earlier studies used PBMCs because EBV DNA is detected in high copy numbers in these cells [33,34,36]. Cell-free EBV DNA is also present, although at lower quantities [67]. Recent studies have used plasma or serum because they are readily obtained and handled. Several studies reported that quantifying cell-free EBV DNA predicted the development of PTLD [59,68,69]. However, serum or plasma samples lack cell-associated virus and therefore plasma loads are not correlated with PBMC values [70]. Stevens et al. reported that the increased EBV DNA loads in PTLD patients were restricted to the cellular compartment, as parallel serum samples were below the cut-off value [67]. Clave et al. also reported that viral DNA was detected only in the cellular compartment in some patients and that measuring EBV load in the plasma could provide a false negative result [62]. More recently, unfractionated whole blood has been used because whole blood can be obtained readily and contains all blood compartments that may harbour EBV. There have been several reports that whole blood is better than plasma/serum when testing PTLD patients [67,70–73]. Based on these observations, whole blood is the preferred specimen for PTLD Table 3), although more thorough studies are needed to resolve this controversy.

PTLD pathogenesis differs between stem cell and solid organ transplants. In stem cell transplantation, donor-derived B cells are the origin of PTLD. Immunosuppression and delayed immune reconstitution, both of which are severe just after conditioning but are subsequently cleared, are the major causes of the disease. Increases in EBV load are seen 2–3 months after stem cell transplantation [60]. Two-thirds of PTLD cases occur within 80 days after stem cell transplantation [74]. The overall incidence of PTLD is relatively low in stem cell transplantation (<1%), although T-cell-depleted transplants or the use of anti-thymocyte globulin greatly increases the risk of PTLD. The high EBV load has some positive predictive and a very good negative predictive value, particularly if the graft was T-cell depleted [59,75]. Most transplantation facilities now monitor EBV load with real-time PCR for high-risk patients undergoing stem cell transplantation [3]. In contrast, recipient-derived B cells are the origin of PTLD in solid organ transplantation, although primary EBV infection from donors occurs occasionally through grafts. Immunosuppression, which is necessary to control rejection, is the main cause of PTLD. Immunosuppression usually must be maintained for the lifetime of the patient. Therefore, the risk of developing PTLD continues for life, although early PTLD (in the first year after transplantation) occurs in cases of primary EBV infection [76]. On the other hand asymptomatic EBV reactivation with high viral load is observed during the extended post-transplant follow-up [77]. The predictive value of the EBV load in solid organ transplantation is less clear, because some recipients have high EBV loads and remain stable for months or years without developing PTLD [78,79]. Measuring viral load appears to be most useful in monitoring patients who were EBV-seronegative before transplantation but are at high risk of developing PTLD [76]. The incidence of PTLD also depends on the type of transplantation: multivisceral has the highest incidence (13–33%), followed by intestinal (7–11%), heart–lung (9.4%), lung (1.8–7.9%), heart (3.4%), liver (2.2%), and kidney (1%) [76].

Differences in the management of PTLD between stem cell and solid organ transplantations are summarised in Table 4. Many laboratories and facilities have proposed that measuring EBV load is a valuable diagnostic and prognostic tool for monitoring PTLD. However, as different systems are used for different samples and patients, it is difficult to determine which EBV load value should be used to identify high-risk patients. For example, in stem cell transplantation, some authors recommend that an EBV load of >300 copies/10⁵ PBMCs is indicative of intervention [66], while others have reported that an EBV load >50,000 copies/mL of serum predicts the development of PTLD [69]. We also proposed that an EBV load >10,000 copies/µg PBMC DNA is
indicative of developing PTLD [60]. This indicates that quantitative values cannot be compared between laboratories; therefore, we have not shown any representative PTLD values in Table 4. Recent studies have indicated that changes in viral load kinetics, rather than single viral load measurements, show a better correlation with organ involvement [80].

### AIDS-related lymphoma

Many different EBV-associated diseases develop in patients infected with human immunodeficiency virus (HIV), including not only diseases of lymphocyte origin but also those of epithelial cell origin, such as oral hairy leucoplaikia. For diseases of lymphocyte origin, opportunistic B lymphoproliferative disorders, Hodgkin’s lymphoma, and non-Hodgkin’s lymphoma (NHL) are more common in HIV-infected patients [81]. Three principal types of NHL are recognised in the HIV setting: (1) sporadic Burkitt’s lymphoma, which develops relatively early in disease, (2) peripheral NHL, which occurs at the late stages, and (3) primary central nervous system lymphoma, which predominately occurs in profoundly immunocompromised late-stage patients [4]. EBV appears to play a pivotal role in the development of AIDS-associated primary central nervous system lymphoma and is frequently associated with virus in the cerebrospinal fluid [81]. Importantly, EBV is rarely detected in the cerebrospinal fluid in HIV-infected patients without primary central nervous system lymphoma.

The EBV copy number in PBMCs increases rapidly after HIV infection, and this increase precedes the decrease in CD4+ T cell counts [47]. The presence of EBV in the blood is significantly associated with lower CD4+ T cell counts, but the EBV load is not correlated with CD4+ T cell counts [82]. Piriou et al. suggested that inter-individual differences in EBV load are maintained after HIV infection, providing evidence for the existence of an individual EBV set point. Thus, currently the significance of measuring EBV load in the blood is unclear. Some authors have suggested that EBV loads may be a useful marker to diagnose EBV-associated NHL [83], but longitudinal studies of EBV load in both PBMCs and serum samples from HIV-infected patients have indicated no specific correlation with the development of NHL [84].

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<tr>
<th>Table 4. Management of post-transplant lymphoproliferative disorder: difference between stem cell and solid organ transplantation</th>
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<td>Anti-thymocyte/lymphocyte globulin</td>
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<td>EBV-specific CTL</td>
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<th><strong>Stem cell transplantation</strong></th>
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Hodgkin’s lymphoma

Approximately 40–50% of Hodgkin’s lymphoma patients are EBV-positive and the disease is etiologically linked to EBV in Western countries [2,3], although the role of EBV in the pathogenesis of the disease is unclear. EBV is maintained in Hodgkin and Reed–Sternberg cells in latency type II with episomal DNA [24]. Hodgkin and Reed Sternberg cells are thought to originate from germinal centre B cells. Compared to blast B cells in PTLD, Hodgkin and Reed–Sternberg cells rarely migrate into the peripheral blood. EBV exists predominantly in the serum or plasma as episomal or naked EBV DNA derived from apoptotic lymphoma cells (Figure 2) [47,85]. Indeed, cell-free EBV DNA is detected in the serum of most patients with EBV-associated Hodgkin’s lymphoma [42]. EBV load in the serum or plasma is correlated with therapeutic responses [86], and EBV positivity in post-treatment samples indicates a poor prognosis [42]. Thus, serum and plasma are optimal samples to monitor Hodgkin’s lymphoma (Table 3).

Nasal NK cell lymphoma

Nasal NK cell lymphoma, while rare in Western countries, is relatively common in East Asia. The primary site of involvement is the nasal cavity, but sometimes similar neoplasms develop in extra-nasal sites [87]. Nasal NK cell lymphoma is almost always associated with EBV [88]. Similar to Hodgkin’s lymphoma, nasal NK cell lymphoma patients have increased amounts of circulating EBV DNA in the plasma or serum [89], potentially because apoptotic proliferating tumour cells release EBV DNA [90]. Before treatment, circulating EBV DNA increases from 10^5 to 10^6 copies/mL, and EBV DNA is correlated with the clinical staging and prognosis [91,92]. These results indicate that plasma EBV DNA is a useful tumour biomarker for the initial evaluation of nasal NK cell lymphoma. As shown in PTLD, unfractionated whole blood may be used instead of plasma, although there is currently no comparative data on plasma and whole blood.

Chronic active EBV infection

Chronic active EBV infection is a rare, life-threatening disease that occurs in children or young adults. This disease is characterised by chronic or recurrent infectious mononucleosis-like symptoms, such as fever, hepatosplenomegaly, persistent hepatitis, and extensive lymphadenopathy [2,3]. There is accumulating evidence that the clonal expansion of EBV-infected T or NK cells plays a central role in the pathogenesis of chronic active EBV infection [93–95]. We proposed that this disease consists of a T cell- or NK cell-type disease, based on the results of PBMC fractionation and subsequent quantitative PCR [96–98]. EBV-infected T or NK cells with a latency type II pattern can evade the host cellular immune system due to the limited expression of viral proteins with reduced antigenicity [27,98,99]. Together with its poor prognosis, some investigators have recommended calling this disease EBV-associated T/NK lymphoproliferative disorder [100].

Patients with chronic active EBV infection have much higher viral loads in their peripheral blood than latently infected individuals [31]. Both PBMCs and plasma (or serum) have been used to estimate viral loads [96,101–103]. EBV-infected T and NK cells migrate into the peripheral blood. Cell-free EBV DNA, derived from apoptotic cells in affected organs, is also present in the serum or plasma. Such cell-free EBV DNA is sensitive to deoxyribonuclease digestion, indicating that it is episomal or naked DNA [98]. Interestingly, some patients do not have cell-free EBV DNA [96]. Compared to Hodgkin’s lymphoma, EBV DNA is more cell-associated in the peripheral blood during chronic active EBV infection (Figure 2). Thus, PBMCs are desirable specimens for diagnostic purposes (Table 3), and in fact PBMCs from most patients with chronic active EBV infection have more than 10^{2.5} copies/μg EBV DNA [96]. A higher viral load in the plasma has been associated with deteriorating clinical status [35,102]. Recently, we analysed chronic active EBV infection patients with stem cell transplantation and found that the plasma EBV load at diagnosis, but not PBMC load, was significantly higher in deceased patients than in living patients [104]. The plasma viral load indicates the amount of EBV-infected cells that are infiltrating organs and may reflect organ damage and therefore prognosis (Table 3).

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is prevalent in southern China, northern Africa, and among Alaskan...
Eskimos. Nearly 100% of anaplastic or poorly differentiated nasopharyngeal carcinomas contain EBV genomes and express EBV proteins [2]. The EBV genome is present in transformed epithelial cells, but not in tumour lymphocytes.

In nasopharyngeal carcinoma patients, EBV DNA is detected in the serum or plasma, but not in PBMCs [43,90]. Deoxyribonuclease sensitivity indicates that most of the cell-free EBV DNA is episomal or naked with some encapsidated DNA [90,105], suggesting that DNA is released primarily not only from apoptotic tumour cells but also from cells undergoing lytic infection (Figure 2). Viral copy number in cell-free blood is an important adverse prognostic factor, as is the persistence or reappearance of high copy numbers of EBV DNA in the serum or plasma [106–108]. Based on these reports, serum or plasma is the preferred sample type to measure EBV DNA in nasopharyngeal carcinoma patients (Table 3).

FUTURE PERSPECTIVES
Measuring EBV load is a routine procedure in high-risk patients undergoing stem cell or solid organ transplantation. Real-time PCR is the easiest and most reliable way to measure EBV load and is, therefore, the most widely used method. However, each transplantation facility monitors EBV load with its own “homebrew” system and there is no consensus on the sample type, sample preparation protocol, or assay units used. Each facility uses different primer/probe designs, standards, and equipment. However, standardisation of these materials is necessary for large-scale studies and international comparisons. Ideally, a standardised kit for measuring EBV DNA will be developed and become commercially available. In contrast to “commercially interesting” viruses, such as HIV type 1, hepatitis B virus, and hepatitis C virus, for which commercial kits are available, EBV is classified as a non-commercially interesting viral target [109]. In the era of expanding transplantation medicine, however, the number of transplants and the intensity of immunosuppression are increasing. Furthermore, effective PTLD treatments, such as anti-CD20 monoclonal antibodies, have been developed. Therefore, the importance of measuring EBV load is increasing. In the near future, we will propose the development of a standardised real-time PCR kit for measuring EBV load to permit large-scale studies and international comparisons.

Obviously, measuring EBV load alone is not sufficiently informative to assess a patient’s status. Additional information on viral gene expression would provide a better assessment of each patient’s condition [39]. Qu et al. examined the expression of EBV-associated genes in solid organ transplant recipients and found that persistent low-load carriers expressed only LMP-2, whereas high-load carriers expressed both LMP-1 and LMP-2 [77]. They used qualitative reverse-transcription PCR to detect EBV-associated genes. Very recently, quantitative methods using real-time reverse-transcription PCR have been used to analyse the expression of EBV-associated genes [110,111]. These quantitative methods will help not only to clarify the pathogenesis of EBV-associated diseases but also to manage patients with high viral loads.

Finally, evaluating EBV-specific cellular immunity is helpful to manage EBV-associated diseases. Human leucocyte antigen class I tetramer analysis is a rapid and direct way to quantify EBV-specific CTL [112]. Using tetramer assays, a number of investigators have combined quantitating the EBV load with serial monitoring of EBV-specific CTL in transplant patients [62,113,114]. High viral loads are predictive of PTLD development only when CTL responses are low or undetectable. Such dual monitoring of EBV load and CTL could improve the clinical predictions of PTLD, although the complexity and cost would also increase [76].

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REFERENCES
Measuring Epstein–Barr virus load


34. Riddler SA, Breinin MC, McKnight JL. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood 1994; 84: 972–984.


Epstein-Barr virus (EBV) loads in peripheral blood lymphocytes from patients with chronic active EBV infection. J Infect Dis 1999; 179: 1012–1015.


